EXHIBIT 12

Migration of Talc From the Perineum to Multiple Pelvic Organ Sites

Five Case Studies With Correlative Light and Scanning Electron Microscopy

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ABSTRACT

Objectives: *Genital talc use is associated with increased risk for ovarian carcinoma in epidemiologic studies. Finding talc in pelvic tissues in women with ovarian carcinoma who have used talc is important in documenting exposure and assessing talc's biologic potential, but tissue-based morphology studies have been rarely reported.*

Methods: *We report five patient cases with documented perineal talc use, each of whom had talc (by both polarized light and scanning electron microscopy) in multiple pelvic sites distant from the perineum. Six negative-exposure control patients were also analyzed.*

Results: *Talc particles were found in exposed patients, typically within two or more of the following locations: pelvic region lymph nodes, cervix, uterine corpus, fallopian tubes, and ovaries.*

Conclusions: *Our report adds new insights into the biologic potential of talc and suggests additional anatomic sites that should be closely examined for talc by oncologic surgical pathologists in the setting of perineal talc use.*

Of great current medical, public health, and medicolegal importance is the epidemiologic association of ovarian malignancy and the use of talc cosmetic products in the genital area. Relevant data from epidemiologic studies have shown a clear excess of women with ovarian malignancy who had used talc in their genital area prior to developing cancer compared with control women.[1-5] In 2006, the data were evaluated by the International Agency for Research on Cancer, which concluded that the data were sufficient to classify the use of talc (not containing asbestos) in the genital area as possibly carcinogenic (class 2B).[6] A recent summary of the epidemiologic data, as they existed cumulatively up to 2017, found that genital talc use may increase ovarian malignancy risk by about 30%.[7] A recent Health Canada assessment[8] resulted in a proposed recommendation that talc meets the criteria under paragraph 64(*c*) of the Canadian Environmental Protection Act and may constitute a danger in Canada to human life or health.

Although the hypothesis about talc and ovarian cancer took its origin, in part, from descriptions of talc in ovarian tissue,[9] the presence of talc in the tissues of the genital tract from women with ovarian malignancy has not been a component or focus of interest in epidemiologic studies. Published histopathologic data showing talc in pelvic organs are very limited. Finding talc in the tissues of exposed patients is part of a larger key principle: the

quantification of foreign material in tissue is critical to assessing the disease occurrence, causality, and severity related to that tissue (reviewed by Abraham[10]). This is perhaps best known for asbestos and mesothelioma or pulmonary fibrosis.[11] The most complete quantification is yielded through the digestion of a tissue sample, because this procedure uses much greater amounts of tissue than could be assessed in a histologic tissue section.[10] The procedure can be used to identify and quantify individual particles by transmission electron microscopy (TEM) or scanning electron microscopy (SEM), which are then characterized by energy-dispersive X-ray analysis (EDX), to verify that their elemental compositions are consistent with a specific type of foreign material exposure.[12] Applying TEM and/or SEM and EDX to tissue sections cut from paraffin blocks also yields meaningful quantification when the concentration of particles in tissue is high enough for this detection.[13,14] This procedure can also show the cellular location where the foreign material resides in a tissue section, for example, exogenous particles in macrophages within lymph nodes.[15] Foreign particulate exposure can be estimated by studying histologic tissue sections under polarized light microscopy, which shows birefringent material, including its size and shape.[16,17] Besides the utility of these methods from a scientific point of view, they have also been applied to medicolegal contexts stemming from injuries in various exposure settings, including asbestos.[10]

Tissue digestion must be paired with a good understanding of local histomorphology to be effective and for its data to be properly evaluated in context. Contamination from laboratory or other sources can potentially complicate tissue digestion procedures, in which the anatomic landmarks are necessarily dissolved in the process. A study by Heller et al[18] was done with tissue digestion and subsequent TEM on ovaries from 24 women having hysterectomy/oophorectomy to treat conditions other than ovarian malignancy. Birefringent particles were found in digestates of all 24 patients by light microscopy and talc in approximately half of the patients by TEM, and talc particle counts were unrelated to reported levels of perineal talc use. This suggested to the authors that unassessed exposures, including infant diapering, might help explain the apparently widespread nature of the finding. Also, even though the authors stated they used talc-free gloves, contamination from laboratory processing sources outside the authors' own environment could have also played a role, given the widespread occurrence of talc in many settings.

In a woman with ovarian carcinoma, looking for talc in benign residual ovarian tissue is a good initial way to find evidence of historical exposure, but in many cases, the ovary is largely replaced by the new growth of tumor, and in such situations, there is often little residual ovary found in resected specimens. A subset of authors from the present study has previously described a case report[15] in which a woman with serous carcinoma of the ovary and who had used talc in her genital area was shown to have talc in three of four examined pelvic lymph nodes. A subsequent recent study by the current authors[19] examined the presence of talc in a series of talc-exposed women with ovarian carcinoma and available pelvic region lymph nodes. This study showed that measurements of talc from digestion of nodes may be adversely influenced by contamination, which may spuriously raise measured talc counts and obscure differences between patients that are related to clinical history and that would otherwise be detectable and significant. Instead, our study demonstrated that polarized light microscopy and in situ SEM/EDX are recommended for the assessment of talc in lymph nodes and, by extension, other exposed tissues as well. The main reason is that in situ SEM/EDX preserves anatomic landmarks and so enables a much better assessment of what is likely to be contamination and what is not.

Until now, the presence of migrated talc in multiple locations in the female pelvis/genital tract in the same patient has not been reported. Such a finding, if present, would add new insights into the potential of talc present in the perineum to enter the upper genital tract and demonstrate the importance of a more careful examination of pelvic tissues from women with epithelial ovarian cancer to correlate with clinical history of talc exposure. We report here a series of five patient cases with documented talc exposure of the genital area and with surgically resected pelvic tissues that were examined by polarized light microscopy, SEM, and EDX for the presence of talc that had migrated from the perineum. These results are compared with examination of surgical material from six patients with ovarian carcinoma who had no genital exposure to talc.

Materials and Methods

Five patient cases were received for consultative purposes, each representing a patient with ovarian carcinoma and a history of perineal talc use. Clinical history, including surgical pathology reports, was provided for each patient with the consultative materials; also, additional history, including surgical history and perineal talc use, was obtained directly from the patients. All patient identifiers, including the 18 recognized Health Insurance Portability and Accountability Act identifiers,[20] were removed from the study data prior to final assembly of

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the data and publication. Histologic H&E-stained slides from the oncologic surgical treatment procedure (typically a total abdominal hysterectomy [TAH]/bilateral salpingo-oophorectomy [BSO] and various other auxiliary procedures) were provided by the outside hospital. All slides were analyzed with an Olympus BH-2 light microscope equipped with polarizing filter capabilities (analyzer and rotating polarizer with specimen slide in between). Each tissue slide was first reviewed to verify the histologic features, tumor type (if present), and tissue site (ovary, cervix, uterus, lymph node, etc). Then, each slide was scanned systematically and completely at ×200 under polarized light, and all birefringent particles were counted that were in the same plane of focus as the tissue. Birefringent particles were counted only if they were located more than a few cell widths' distance deep relative to the surface to avoid including any surface contamination in the analysis. Birefringent particles such as paper, organic debris, starch, and other clearly recognizable contaminants were not counted if they were in any way interpretable as related to the surface.

Paraffin blocks corresponding to histologic slides of interest were obtained from the treating hospital. The tissue blocks were handled with a procedure for in situ SEM/EDX, which was first described by Thakral and Abraham,[13] for assessment of particulate materials in paraffin-embedded tissue. The full details of this procedure as it was applied in our laboratory are available elsewhere.[21] Importantly, to protect against contamination, the tissue blocks were handled with particle-free gloves on precleaned surfaces and sectioned removing ~30 μm of tissue and paraffin using a rotary microtome with a fresh, clean stainless-steel blade. This sectioning was intended to remove any surface contamination from previous storage and handling. After the fresh surface was exposed, the block surfaces were washed in distilled, deionized water for 30 seconds to remove soluble surface materials such as sodium chloride and sodium phosphates used in processing for histology. The blocks were mounted for SEM examination and always kept in closed containers to limit any environmental contamination. A Hitachi SU6600 field emission SEM was used, with an Oxford EDX with Aztec version 2.0 to 3.3 software, EDX detector model X-Max 50 SDD, and electron beam penetration depth estimated at 2.5 μm, with an X-ray microanalysis range of 0.5 to 2.5 μm in depth. Talc particles were characterized by magnesium (Mg) and silicon (Si) peaks falling within 5% of the theoretical atomic ratio of 0.750 and atomic weight percent ratio of 0.649 (representative talc spectrum is shown in **Image 1**).

Because all patients in this study were born before 1970, which was the time point when talc manufacturers

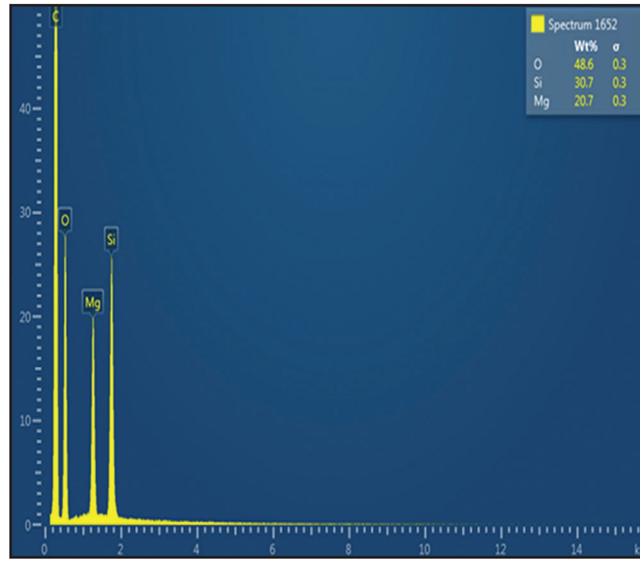

Image 1 Representative spectrum of talc, showing characteristic magnesium (Mg) and silicon (Si) peaks. The characteristic Mg-Si atomic ratio is 0.75 and atomic weight percent ratio is 0.649, and particles are considered to be talc if their Mg-Si ratio falls within 5% of this theoretical value (0.649).

claimed to voluntarily remove asbestos contamination from commercial talc preparations (establishing a cosmetic grade "free of asbestos" vs industrial grade that may contain it),[22,23] and because these patients had talc exposure extending across many years, we re-reviewed all SEM backscattered electron images generated on each patient specifically for fibers or fiber-like particles (defined as a 5:1 aspect ratio). We separately tallied and categorized them (one caveat being that plate-like particles, when viewed on edge, could give the impression of being a fiber, whereas with another orientation, they might not). The EDX spectrum for any particle meeting the fiber criterion was reexamined to determine its chemical composition, and where necessary, atomic weight percent calculations were done to determine fit (or lack of fit) with known classes of inorganic fibers.

To provide a set of nonexposed controls for the five patients in this case series, six patients with ovarian carcinoma were identified (see Results section and Supplementary Table 1; all supplemental materials can be found at *American Journal of Clinical Pathology* online) who were part of a large case-control study of ovarian cancer in eastern Massachusetts and New Hampshire.[3] Patients completed structure interviews and provided written informed consent allowing for review of pathologic material from their surgery. The study was approved by the Dana Farber/Harvard Cancer Center Ethical Review Panel. Patients were selected who stated that they had not used talc, either in their perineal area or as a general body

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powder. These patients had a distribution of tumor types (five serous carcinomas, one endometrioid carcinoma), ages (47-58 years), and remote surgical history (ie, prior to the development of cancer) similar to the five patients in the main study, and all had undergone TAH/BSO as part of their surgical oncologic treatment. In addition, patients from the Brigham and Women's Hospital were selected to facilitate retrieval of archival materials. H&E slides were examined by regular and polarized light microscopy. A count of birefringent particles was made by systematic, complete review at ×200 of each H&E slide under polarized light microscopy, the same as for the patient slides in the main study. Subsequently, and also similar to the main study, tissue blocks were examined with SEM/EDX, using the same in situ method previously described, and with all talc or other backscattered electron imaging-positive particles characterized.

Results

Table 1 shows key clinical details of the five talc-exposed women in this series. Ages fell within a fairly narrow range (47-59 years). Three patients had serous carcinoma, one endometrioid carcinoma, and one clear cell carcinoma. All these histologic types have been identified as being included in the general increase in risk with talc exposure in epidemiologic studies.[5] Pathologic International Federation of Gynecology and Obstetrics staging ranged from IA (one patient) to IC (one patient) to IIIC (three patients). All patients had BSO, and four of the five had accompanying TAH. Four of five had pelvic region lymph nodes excised for staging and/or treatment purposes.

Table 2 shows the findings from polarizing light microscopy of key sections from the talc-exposed patients' resected tissues. All patients had significant numbers of birefringent particles in tissue sections from two or more pelvic region sites, ranging from two (case 1, exocervical soft tissue and right pelvic lymph node) to four (case 2, with large numbers of particle accumulations in uterine serosa, pelvic lymph nodes, ovaries [right > left], and the fibromuscular tissue surrounding the right fallopian tube). Case 3 showed birefringent particles in the uterine serosa, bilateral fallopian tubes, and ovaries. Cases 4 and 5 had birefringent particles in the tissues of multiple

Table 1
Talc-Exposed Patients' Clinical Histories

Case No.	Age, y	Tumor Type	Pathologic Stage	Surgical Procedure Type	Exposure History (Talc Years)[a]	Comments
1	47	Endometrioid carcinoma, G3 (poorly differentiated)	pT1c N0 MX (FIGO stage IC)	TAH/BSO with multiple pelvic/para-aortic lymph node excisions, with omentectomy/appendectomy (February 2009)	42	No history of surgeries prior to TAH/BSO
2	50	Serous carcinoma, high grade	pT3c N1 MX (FIGO stage IIIC)	TAH/BSO with multiple pelvic/para-aortic lymph node excisions, with omentectomy (October 2013)	31	Diagnostic cervical, pelvic mass, and pelvic lymph node biopsies performed a few months prior to TAH/BSO; tubal ligation at age 20 years
3	59	Serous carcinoma, high grade	pT3c NX MX (FIGO stage IIIC)	TAH/BSO with omentectomy (June 2010)	58	Diagnostic omental mass biopsy 2 weeks prior to TAH/BSO; tubal ligation and cesarean section at age 32 years and cholecystectomy at age 45 years
4	49	Serous carcinoma, low grade	pT3c N0 MX (FIGO stage IIIC)	BSO with multiple pelvic/para-aortic lymph node excisions, with omentectomy, appendectomy, and right hemi-diaphragmectomy (March 2013)	31	No history of surgery prior to BSO
5	56	Clear cell carcinoma, grade 2	pT1a N0 MX (FIGO stage IA)	TAH/BSO with multiple pelvic/para-aortic lymph node excisions, with omentectomy/appendectomy (March 2009)	51	No history of surgery prior to TAH/BSO

BSO, bilateral salpingo-oophorectomy; FIGO, International Federation of Gynecology and Obstetrics; TAH, total abdominal hysterectomy
[a]Talc year = daily (at least) application of talc-containing hygiene product to the genital area for 1 year. Patients 3 and 5 had reportedly experienced talc exposure since birth and/or early in infancy.

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pelvic sites (fallopian tubes, ovaries, pelvic region lymph nodes), but due to the logistics of case review, processing, and send-out, we were not able to quantify these retroactively with additional light microscopy after the in situ SEM data had been obtained on the same blocks.

Table 3 shows in situ SEM/EDX data on the same patients as in Table 2, with 28 total blocks (across the five patients) examined by SEM/EDX and included in our case series (most, but not all, of the blocks in which birefringent particles were seen subsequently proceeded to electron microscopy). As is shown, there were generally substantial talc particle counts in the same tissue blocks corresponding to where birefringent particles were identified by light microscopy. For example, markedly high light microscopic particle counts in Table 2 for case 1 (cervix and right pelvic lymph node), case 2 (right fallopian tube), and case 3 (uterine serosa) were all matched by high talc particle counts by SEM/EDX for the corresponding cases and tissues in Table 3. Comparative examination of the data in Tables 2 and 3, particularly the pairs

Table 2
Polarizing Light Microscopy Findings in Pelvic Tissues From Five Talc-Exposed Patients

	Distribution of Birefringent Particulates Within Tissue (Particles/Histologic Section), No.			
Case No.	Lower Tract (Uterus/Cervix)	Fallopian Tube	Ovary	Lymph Nodes
1	Cervix: >100	Left tube: 3 Right tube: 3	Left ovary: 6	Right pelvic node: >500 Left pelvic node: >50
2	Anterior cervix: 6 Posterior uterus: >50	Right fallopian tube: >50, mainly in fibromuscular tissues near the tube	Right ovary, first block: 13 Right ovary, second block: 35 Right ovary, third block: 3 Left ovary, first block: 1 Left ovary, second block: 6	Right pelvic node: >100 Left pelvic node, first block: >200 Left pelvic node, second block: >100 Left pelvic node, third block: >100 (Note: first through third blocks are together one node.)
3	Uterus: >200	Right tube: 15 Left tube: 14	Right ovary: 27 Left ovary: 11	None surgically resected
4	Tissue type not made available	Birefringent particles seen in left fallopian tube (one block) and right fallopian tube (two blocks); exact counts not available	Birefringent particles seen in left ovary (two blocks) and right ovary (two blocks); exact counts not available	Birefringent particles seen in right pelvic lymph nodes (four blocks) and left pelvic lymph node (one block); exact counts not available
5	No birefringent particles seen in tissues	Birefringent particles seen in left fallopian tube (one block) and right fallopian tube (one block); exact counts not available	Birefringent particles seen in right ovary (one block) and left ovary (one block); exact counts not available	Birefringent particles seen in left pelvic lymph nodes, two blocks (three nodes total by gross examination report); exact counts not available

Table 3
In Situ Scanning Electron Microscopy Findings for Pelvic Tissues in Five Talc-Exposed Patients

	No. of Talc Particles Found in Each Tissue Block by In Situ Scanning Electron Microscopy/EDX			
Case No.	Lower Tract (Uterus/Cervix)	Fallopian Tube	Ovary	Lymph Nodes
1	Cervix: 52	Left tube: no SEM done Right tube: no SEM done	Left ovary: 8	Right pelvic node: 65 Left pelvic node: 61
2	Anterior cervix: 1 Posterior uterus: 53	Right tube: 31	Right ovary, first block: 2 Right ovary, second block: 51 Right ovary, third block: 0 Left ovary, first block: 1 Left ovary, second block: 3	Right pelvic node: 18 Left pelvic node, first block: 43 Left pelvic node, second block: 35 Left pelvic node, third block: 24 (Note: first through third blocks are together one node.)
3	Uterus: 36	Right tube: 2 Left tube: 1	Right ovary: 24 Left ovary: 0	None resected
4	Not examined by SEM	Not examined by SEM	Right ovary, first block: 4 Right ovary, second block: 0	Right pelvic node: 28 (one block examined)
5	Not examined by SEM	Right tube: 0	Right ovary: 8 Left ovary: 0	Left pelvic node: 13 (one block examined)

EDX, energy-dispersive X-ray analysis; SEM, scanning electron microscopy.

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of tissue blocks (generally in cases 1 through 3) for which both polarized light microscopy and SEM/EDX numerical particle counts were available, showed an *r* value of 0.675 and a *P* value of .002 by linear regression analysis. Where direct comparisons between the counts in Tables 2 and 3 were able to be made (generally the first three cases), counts of birefringent particles by light microscopy were generally higher than the corresponding counts by SEM. This can be explained by the finding of other nontalc foreign material in the blocks by SEM/EDX. EDX analyses were performed of backscatter-positive particles seen in these blocks. Across the 28 blocks, this yielded an aggregate total of 503 talc particles and 945 foreign nontalc particles. Of the latter, most (802, 85%) were nonspecific mineral particles consisting generally of Si in various combinations with sodium (Na), Mg, and especially aluminum (Al). Where Mg and Si predominated in this group, the spectral peak ratio fell outside the atomic weight percent range (0.649% ± 5%) expected for talc, so they were not classified as such. Occasionally, silicon oxide particles were identified by SEM/EDX, which exhibits birefringence.[17] The remaining exogenous particles consisted of various metals, either alone or in various combinations, most notably copper, chromium, Al, titanium, zinc, nickel, and manganese. Iron (Fe) was often combined with some of these metallic particles. Besides talc and exogenous metals and minerals, the other broad category of particles seen in the case analyses included endogenous particles, often in the form of dystrophic calcification, which is common in serous ovarian malignancy. Particles with calcium (Ca), Na, phosphorus (P), carbon, potassium (K), and Fe in various combinations were considered endogenous. No asbestos fibers or ferruginous bodies were found in the analyses. Based on the data, the nonspecific mineral particles accounted for many of the birefringent particulates seen under light microscopy that were not talc. Such particles can be encountered in everyday living and may presumably gain access to the perineum and associated lymphatics in similar ways to talc. Based on data from Jurinski and Rimstidt[24] for talc vs silica, these nonspecific silicates could be reasonably expected to have a slow dissolution rate (years or decades) and a long retention time in tissue.

Tissue macrophages were a key particle location for many sites and thus a key part of the tissue response to the migrated talc. Such cells, with cytoplasm filled with birefringent particles, were seen in the cervix (case 1) and the uterine serosa and pelvic region lymph nodes of multiple cases. In rare instances, the affected macrophages were seen to coalesce into multinucleate giant cells as part of the inflammatory response. Affected macrophages often had grayish, faintly ground-glass cytoplasm and were sometimes accompanied by a mix of other chronic inflammatory cells (eg, in the soft tissues near the fallopian tube in case 5). Birefringent material was often seen localized near small vasculature, particularly in the uterine serosa (cases 1 and 3) and soft tissue near fallopian tubes (cases 2 and 5). For extranodal talc migration sites, the concomitant presence of lymphatic vessels was strongly suspected, but this was often difficult to ascertain histologically since these vessels may be nonpatent and/or otherwise hard to see in tissue sections. Of note, for three of the four patients who had pelvic region lymph nodes resected, none of their talc-positive lymph nodes had concomitant metastatic malignancy. However, case 2 had two pelvic region lymph nodes (represented on slides as multiple lymph node profiles) with both metastatic tumor and abundant birefringent particles in macrophages, often existing close to each other. An example is shown in **Image 2**. Because both are regarded as migrating via lymphatic pathways, their coexistence in one of our patient cases was not a surprise. **Image 3** emphasizes this point from a different point of view by showing regular H&E (Image 3A) and polarized light microscopy (Image 3B) of the same view of uterine serosa in case 3. Several lymphovascular spaces are present, a larger one of which is highlighted with the arrows and is seen to contain birefringent material in Image 3B. EDX of this patient's uterine serosa tissue showed that this birefringent material was talc (see next paragraph).

Image 4, **Image 5**, **Image 6**, **Image 7** and **Image 8** (pertaining respectively to cases 1 through 5 in Tables 1-3) show representative correlative polarized light and SEM (with backscattered electron imaging) micrographs. For each case, EDX analysis of most of the backscattered image-positive particles (typically 1-10 μm diameter) showed the characteristic spectrum of talc in Image 1, thus confirming that most of the birefringent material seen by polarizing light microscopy in these particular areas was, in fact, talc. Considering each figure individually, Image 4 shows birefringent material clustered in macrophages in deep exocervical fibrous tissue and comparable particle morphology in the same region on backscattered electron SEM imaging. Similar correlative morphology is seen in the same figure for macrophages within pelvic lymph node tissue. The exocervical tissues and lymph node show rather unremarkable macrophage morphology when reviewed by light microscopy without polarization. Image 5 shows birefringent particle accumulations in the uterine serosa (both macrophages and soft tissue and near vascular spaces) and fallopian tube peripheral tissue, ovary, and lymph node tissue (the latter frequently in macrophages), with corresponding morphology in the SEM backscattered electron images. Image 6 shows

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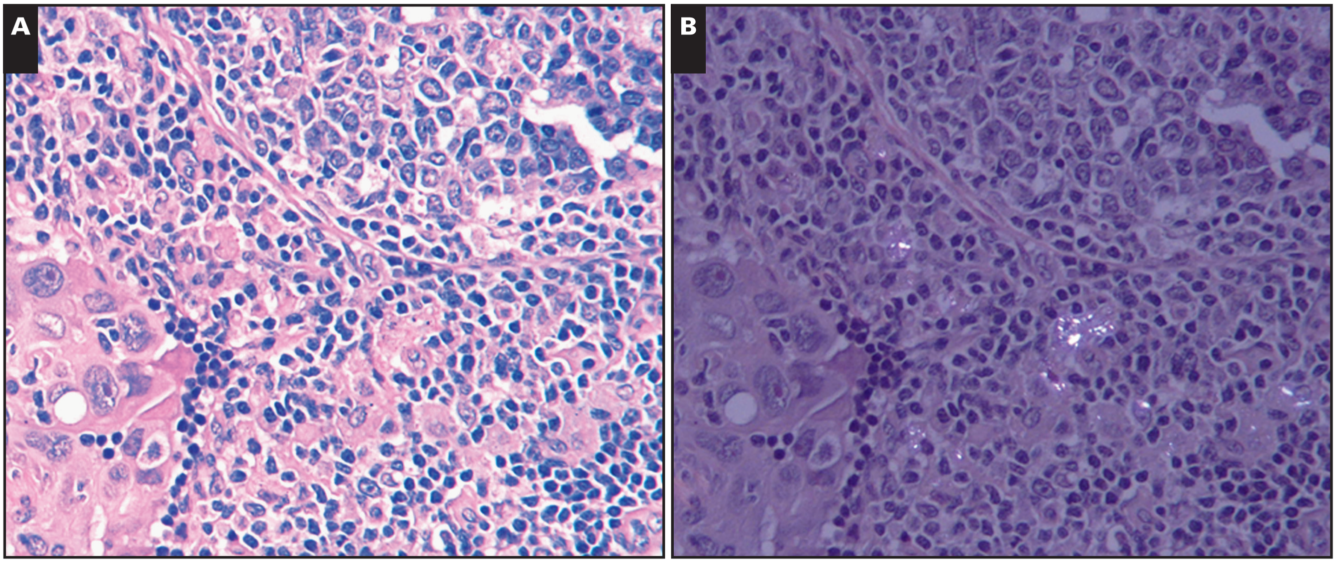

Image 2 Regular light microscopy (**A**) and polarized light microscopy (**B**) of left pelvic lymph node in patient 2, same field of view, showing juxtaposition of birefringent particles in macrophages, metastatic carcinoma, and uninvolved lymph node parenchyma. This particular area was not analyzed by scanning electron microscopy, but based on the findings in other histologic regions, much of this birefringent material is likely talc. (H&E, ×400)

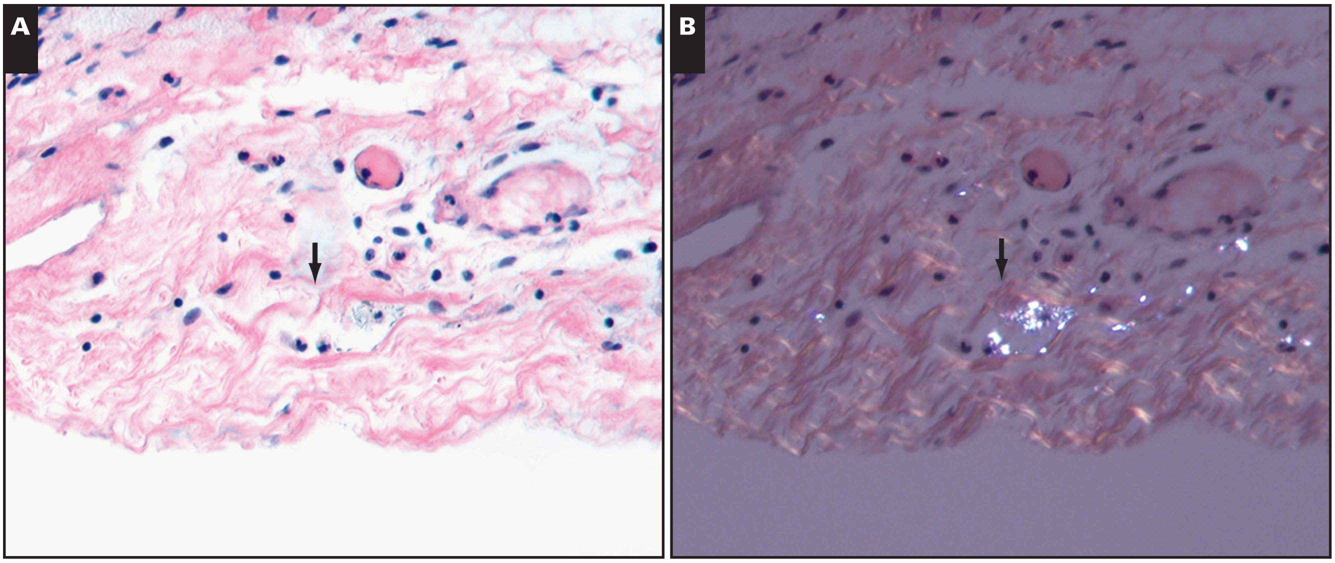

Image 3 Regular light microscopy (**A**) and polarized light microscopy (**B**) of the uterine serosa in patient 3, same field of view, showing serosal fibrovascular tissue and abundant birefringent particles that are seen in one lymphvascular space (arrows). This serosal birefringent material was shown to be talc by scanning electron microscopy/energy-dispersive X-ray analysis (see Image 6). (H&E, ×200)

uterine serosa with numerous birefringent particles within soft tissue and macrophages, as well as ovarian stroma showing a birefringent particle within soft tissue but close to a blood vessel, with corresponding SEM images showing backscattered electron-positive particles. Images 7 and 8 show birefringent particles in pelvic region lymph nodes with corresponding backscattered electron-positive positive SEM images, as well as birefringent particle(s) in auxiliary sites: ovary (Image 7) and soft tissue from around the fallopian tube (Image 8). The latter was notable for a mixed chronic inflammatory infiltrate in and around the exogenous material.

Review of the backscattered electron SEM images from all 28 tissue blocks from all five patients (typically there were around 50-250 SEM images generated on each block) showed a total of 52 fibers or fiber-like particles,

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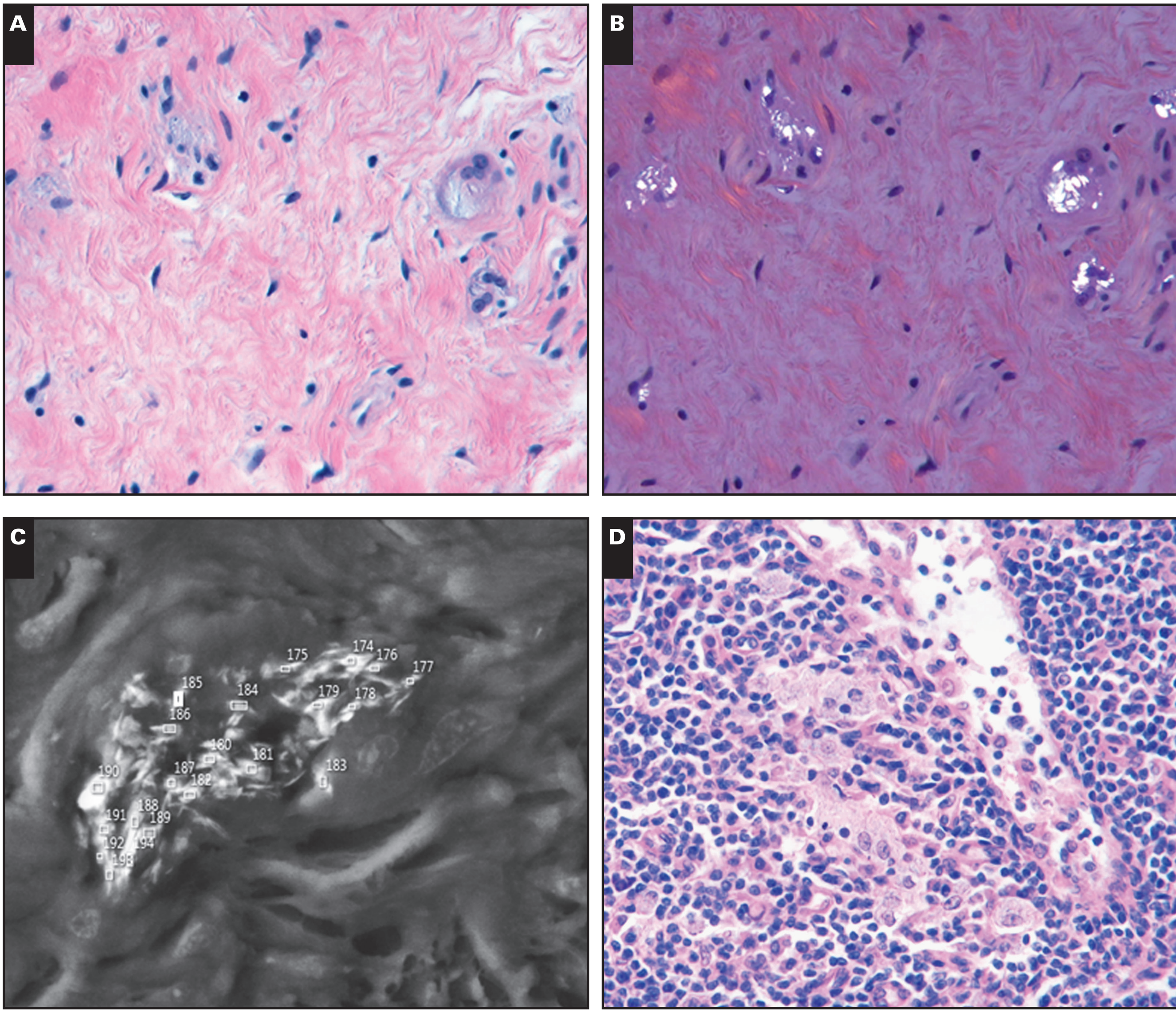

❚Image 4❚ Representative photomicrographs for patient 1. **A**, Deep exocervical soft tissue with collections of macrophages in dense collagenous tissue. A few macrophages were multinucleate and showed slightly glassy grayish cytoplasm (H&E, ×400). **B**, Same histologic field as **A**, under polarized light microscopy, showing collections of macrophages with numerous birefringent cytoplasmic particles 1 to 10 µm in diameter (H&E, ×400). **C**, Scanning electron microscopy (SEM, ×500) with backscattered electron imaging from the same general area as in **A** and **B** but a different histologic section, showing numerous backscattered electron-positive particulates within the cytoplasm of macrophages, similar to **A**, the majority of which had a spectrum characteristic of talc. **D**, Right pelvic lymph node with aggregates of intranodal macrophages (H&E, ×400).

of which 18 (35%) were talc, 18 (35%) were nontalc mineral silicates (typically Al-Si often in combination with other cations), six (11%) were metals or combinations of metals, and 10 (19%) were endogenous (various combinations of Na, P, sulfur, Ca, K, and Fe). Most of the identified fiber-like particles had aspect ratios approximately or slightly greater than 5:1 (the threshold we used), but there were four fibers identified with long aspect ratios (>10:1) and strongly parallel sides. Three of these were found in the right ovary of patient case 2, and the fourth was found in the left fallopian tube of patient case 3. By EDX, these fibers were aluminum silicates with Mg and Ca and, in two of the fibers, also Fe. Atomic weight percent calculations on these fibers showed that the Mg/Si and Ca/Si ratios were far outside the ranges expected for tremolite asbestos fibers; also, the presence of Al was additional evidence against tremolite since it would not be expected to occur in the latter. Asbestos fibers or ferruginous bodies, if present, were below the level of detection of our analysis and

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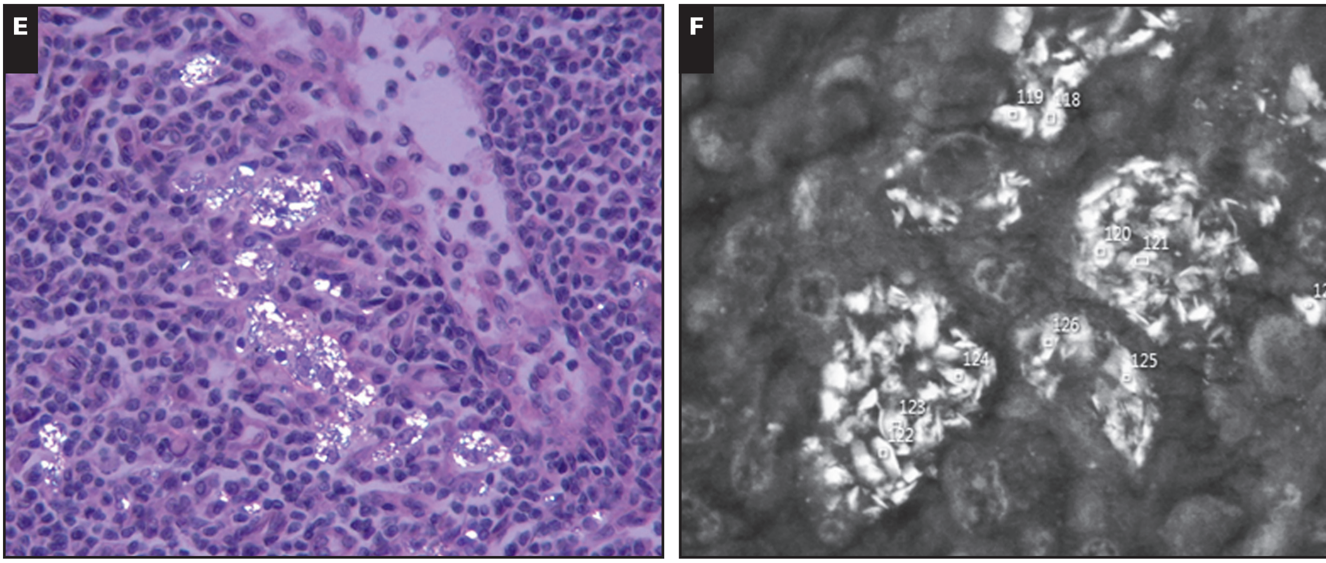

▌Image 4▌ (cont) **E**, Same histologic field as **D**, under polarized light microscopy, with numerous birefringent particles similar in size and appearance to those seen in the exocervix, within intranodal macrophages (H&E, ×400). **F**, SEM (×500) from the same general area as **D** and **E** but in a different histologic section, showing numerous backscattered electron-positive particulates within the cytoplasm of macrophages, similar to **C**, and the majority having a spectrum characteristic of talc.

hence not found. A representative sampling of the fibers and fiber-like particles we found is shown in **▌Image 9▌**, along with more details on the atomic weight percent calculations.

Supplementary Table 1 shows the clinical, light microscopic, and SEM data for the six control patients with no history of perineal or body use talc exposure. Ten ovary blocks and one fallopian tube block comprised the six control patients' materials. Polarizing light microscopy, as shown in the table, revealed a range of two to 17 birefringent particles per slide; these values are comparable to the lower end of the polarizing light microscopy results of the exposed patients in Table 2 but markedly less than for tissues from those patients who had substantial talc by subsequent SEM/EDX. Inflammatory infiltrates, when seen in the control tissues, were generally attributable to the presence of nearby tumor and not to the presence of the uncommon birefringent material. Giant cells, such as were seen in some talc-exposed patients, were not observed in controls.

Correlative SEM/EDX of the control tissue blocks showed a total of four talc particles across all patients: two in patient 2 (right ovary) and two in patient 3 (right fallopian tube). Of note, in Supplementary Table 1, both these patients had pelvic surgery more than 30 years prior to their ovarian cancer surgical procedure. The talc particles represented a very small proportion (0.8%) of the overall backscattered electron-positive particles that were found and analyzed across the 11 control tissue blocks (494). Of those, most were endogenous, the most common being calcium phosphate (202 [41%] particles), sodium salts (108 [22%] particles), and iron phosphate (56 [11%] particles). Nonspecific minerals accounted for 105 (21%) particles; these may access the genitourinary tract through hygiene practices and general living. No fibers, talc or otherwise, were found in any control tissues by SEM/EDX.

Discussion

The cases reported here show in vivo pelvic migration potential for talc that has, to our knowledge, not been reported previously. Within a set of five patient cases, all with known talc exposure to the perineum and all of which had groups of pelvic organs/tissues surgically resected for the management of ovarian carcinoma, talc was found in two pelvic organ sites (three patients), three sites (one patient), and four sites (one patient) distant from the original site of application (perineum). In four of the five patients, pelvic region lymph nodes were one of the sites affected. Talc has been described in one distant pelvic organ site before[15,19] but, prior to this report, not more than one such site in a given patient.

It is important to remember that, because the in situ SEM technique examines only a very small volume of tissue, the finding of even modest numbers of exogenous particles (eg, talc) in tissue sections may translate into a

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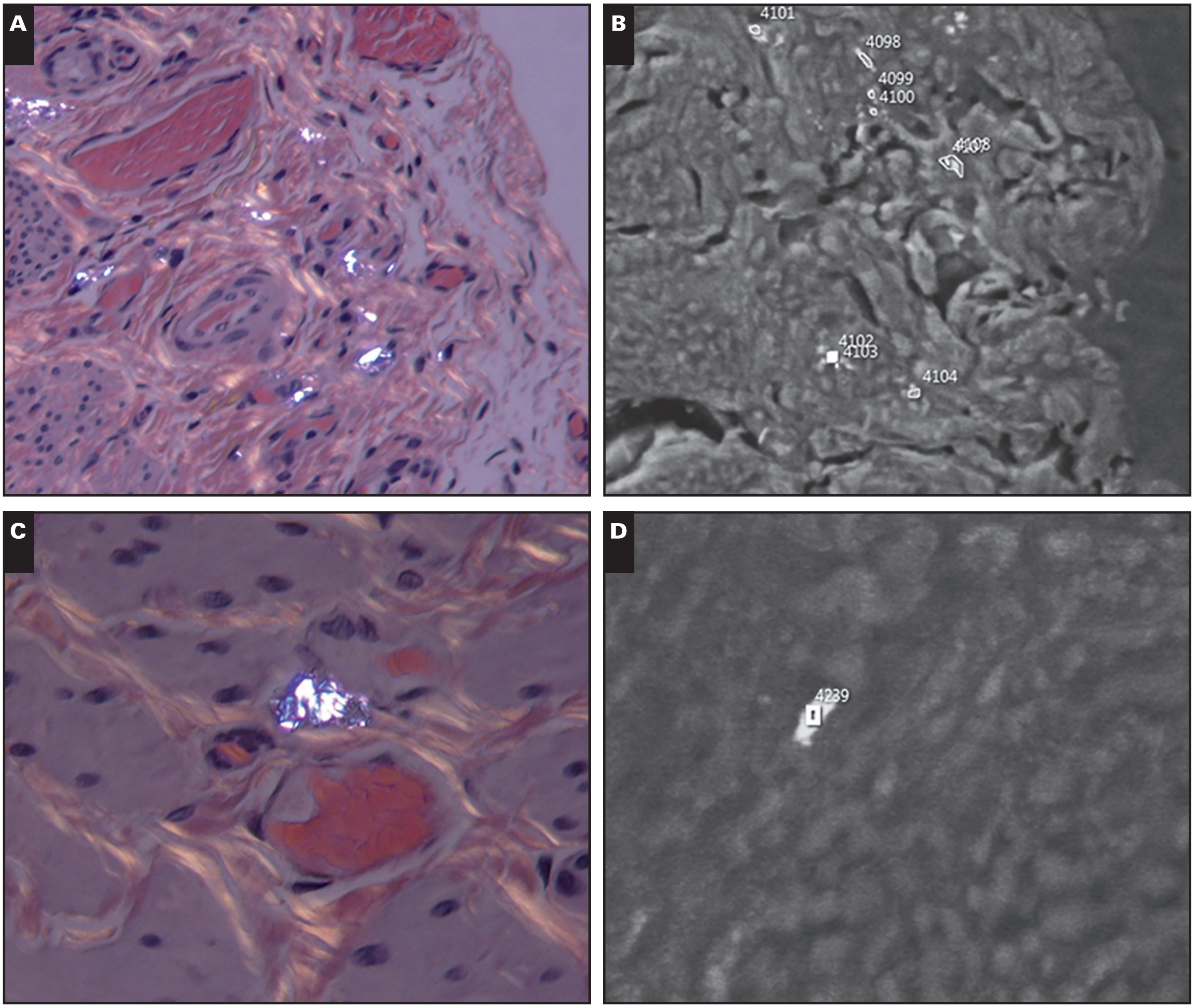
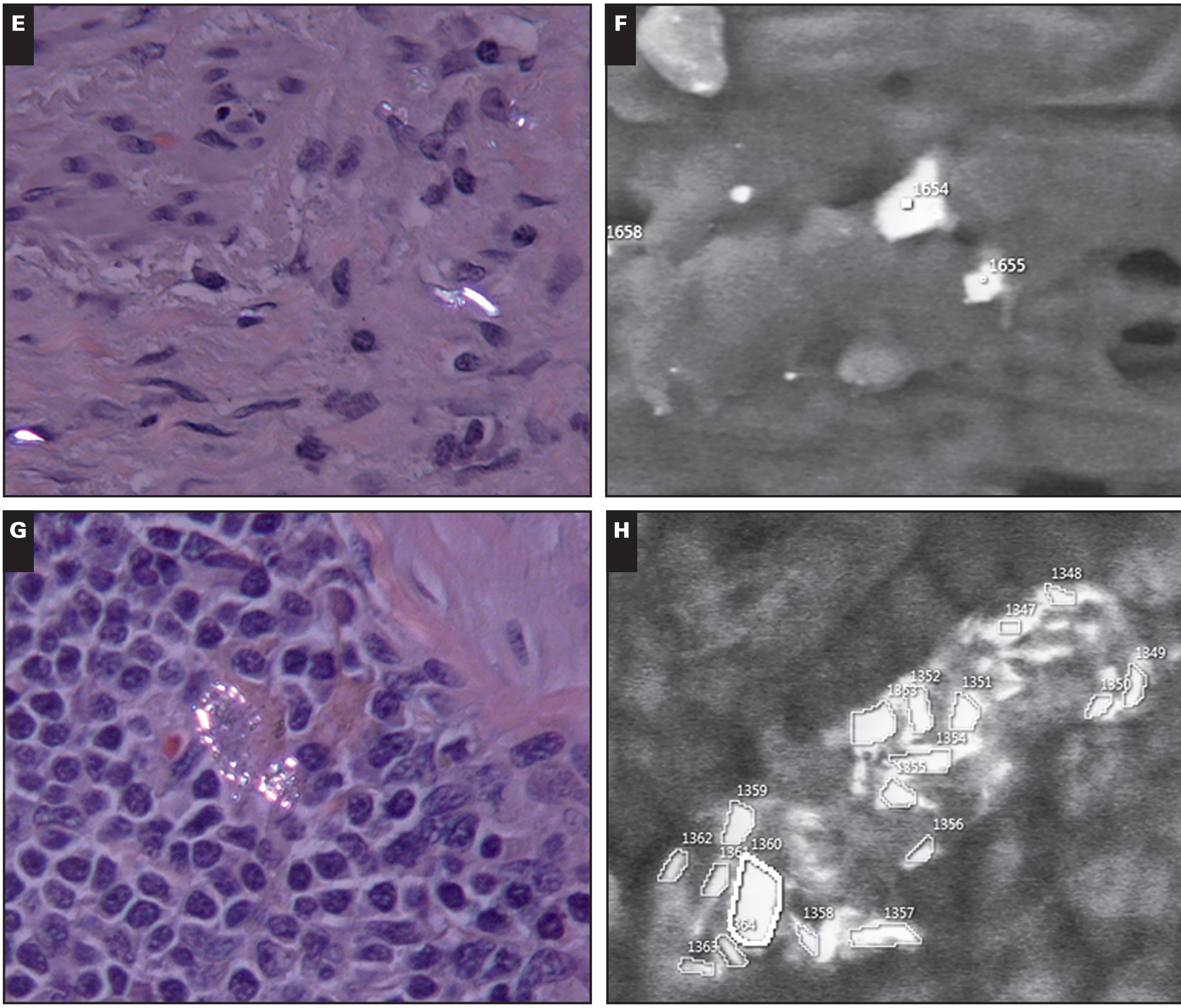


Image 5 Representative photomicrographs for patient 2. **A**, Uterine serosa showing numerous birefringent particles 1 to 10 mm in diameter within soft tissue and macrophages (H&E, ×200). **B**, Scanning electron microscopy (SEM) corresponding to **A**, showing numerous backscattered electron-positive particles (×500), the majority with an energy-dispersive X-ray analysis (EDX) spectrum characteristic of talc. **C**, Fibromuscular soft tissue near fallopian tube, showing a macrophage with abundant intracellular birefringent material similar to that seen in **A** (H&E, ×400). **D**, SEM of the same region as **C**, showing a backscattered electron-positive particle approximately 5 μm in diameter that proved to be talc using EDX (×500).

significant exposure when calculated on a per-gram-of-tissue basis and when placed in appropriate clinical context. Or, to put it another way, seeing particles by in situ microscopy (both light and SEM) requires a relatively large amount of material distributed within the tissues to make it possible to find it in this manner. Roggli and Pratt[25] demonstrated this principle by showing that the identification of one asbestos body in a tissue section corresponded to at least 100 fibers per gram of tissue.

The six control cases supported the contention that talc is rarely found in surgically resected pelvic tissues from patients with no prior perineal or body use exposure. The four talc particles found by SEM/EDX were in two patients who had undergone pelvic surgical procedures more than 30 years prior. Given that history and timeline, the talc could have been introduced from the ambient environment or from talc on instruments or gloves. The latter was relatively common decades ago when these patients had surgery.[26] Birefringent particles of other etiologies (endogenous or nonspecific mineral particles) can be found in nontalc-exposed patient tissues, as was the case in our controls, with SEM/EDX useful in



▌Image 5▌ (cont) **E**, Birefringent particle seen in soft tissue of ovary, with mixed inflammation and fibrosis in the general background (H&E, ×400). **F**, SEM showing several backscattered electron-positive particles within the same region as **E** (ovary). The particles were irregularly shaped, less than 7 μm in diameter, and on EDX analysis showed the characteristic spectrum of talc (Image 1) (×500). **G**, Left pelvic lymph node with numerous birefringent particles similar in size and appearance to those seen in the uterine serosa, within intranodal macrophages (H&E, ×400). **H**, SEM from the same general area as **E** but in a different histologic section, showing numerous backscattered electron-positive particulates within the cytoplasm of macrophages, similar to **E**, with most having an EDX spectrum characteristic of talc (×500).

the distinction. However, most of the numerous calcium phosphate particles found in the controls would likely have been nonbirefringent and thus not detected by polarizing light microscopy.

The five cases described here were part of a larger group of cases (all women with ovarian carcinoma and with perineal talc exposure) that were received by us for consultative purposes over a 3- to 4-year period. Among 34 consults recently reviewed by one author (S.A.M.), 29 (85%) had birefringent particles in more than one pelvic organ site, and of the five that did not, three had substantially limited material for review. Most of these cases have not yet had SEM/EDX performed on tissue blocks, so we do not yet know to what extent these light microscopic findings translate into sites of talc migration. But these preliminary data suggest that a substantial fraction might among patients with the appropriate exposure history.

A prominent finding in several of our cases and tissue sites was the accumulation of numerous birefringent particles in the cytoplasm of tissue macrophages

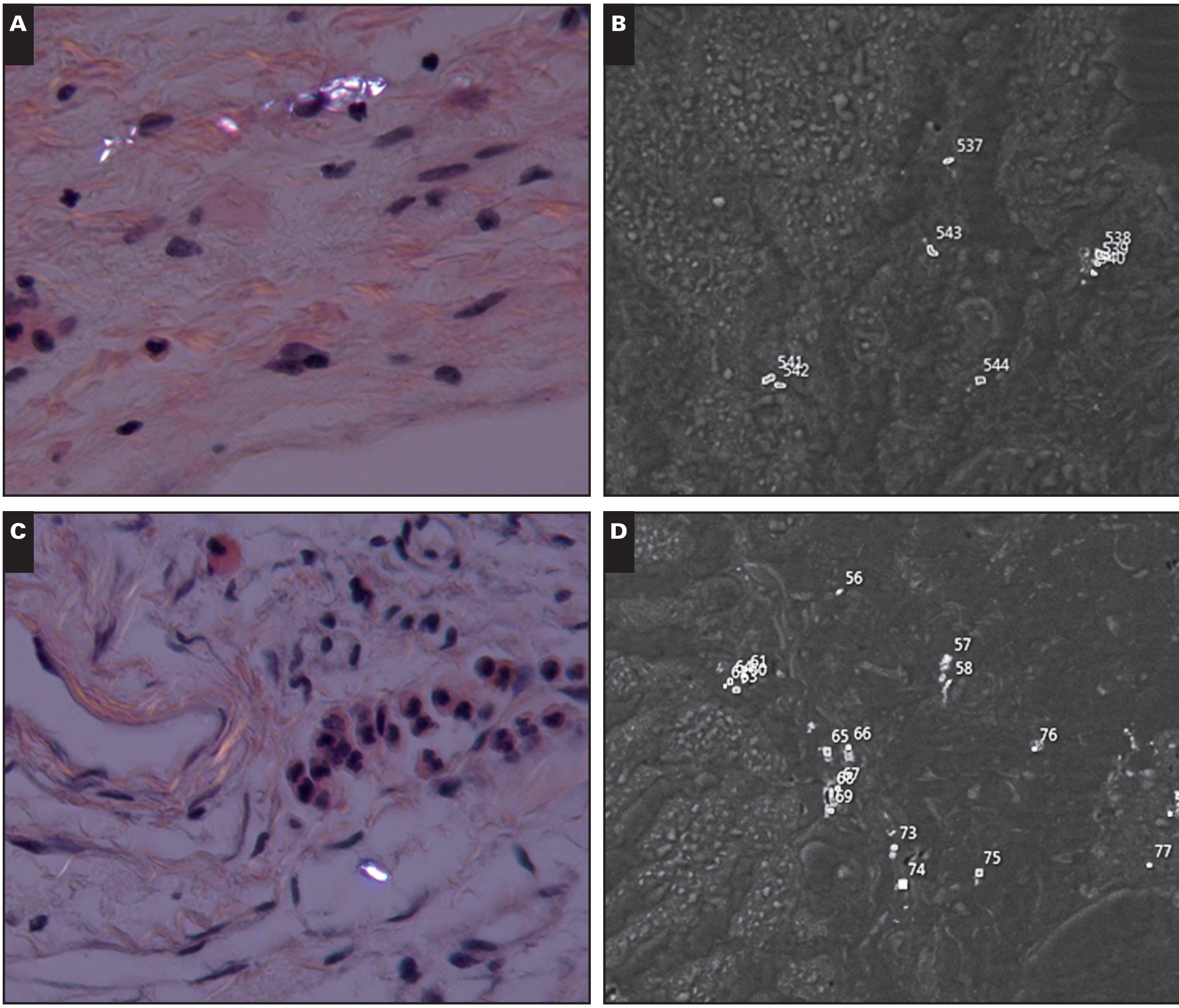


Image 6 Representative photomicrographs for patient 3. **A**, Uterine serosa showing numerous birefringent particles 1 to 10 μm in diameter within soft tissue and macrophages (H&E, ×400). **B**, Scanning electron microscopy (SEM) corresponding to **A**, showing numerous backscattered electron-positive particles (×500), most with an energy-dispersive X-ray analysis spectrum of talc similar to Image 1. **C**, Ovarian stroma showing a birefringent particle within soft tissue but close to blood vessel. Other birefringent particles were seen in different microscopic fields in this section. **D**, SEM showing several backscattered electron-positive particles within the same tissue (but different section) corresponding to **C**. These backscattered electron-positive particles showed a spectrum characteristic of talc (Image 1) (×500).

on both light microscopy and SEM, which, using EDX analysis, proved to be talc. The H&E appearance of these macrophages was often rather subtle, with grayish cytoplasm and a faintly ground-glass appearance; in our opinion, they could be easily missed on a routine slide review where just light microscopy is performed. This therefore highlights the importance of doing polarizing light microscopy on surgically resected pelvic tissues, not necessarily in every case but indeed if or when the appropriate talc exposure history is present.

Talc is able to stimulate the phagocytic potential of macrophages: a subset of the current authors and their colleagues reanalyzed the slides from the study by Beck et al,[27] who did in vivo hamster studies using sonicated intratracheally induced talc and granite exposure. It was found that pulmonary macrophages phagocytize talc more avidly than granite, especially in the initial 1 to 2 days following exposure (unpublished data). Beck et al[27] showed that these macrophages that have ingested talc are then unable to phagocytose radiolabeled particles

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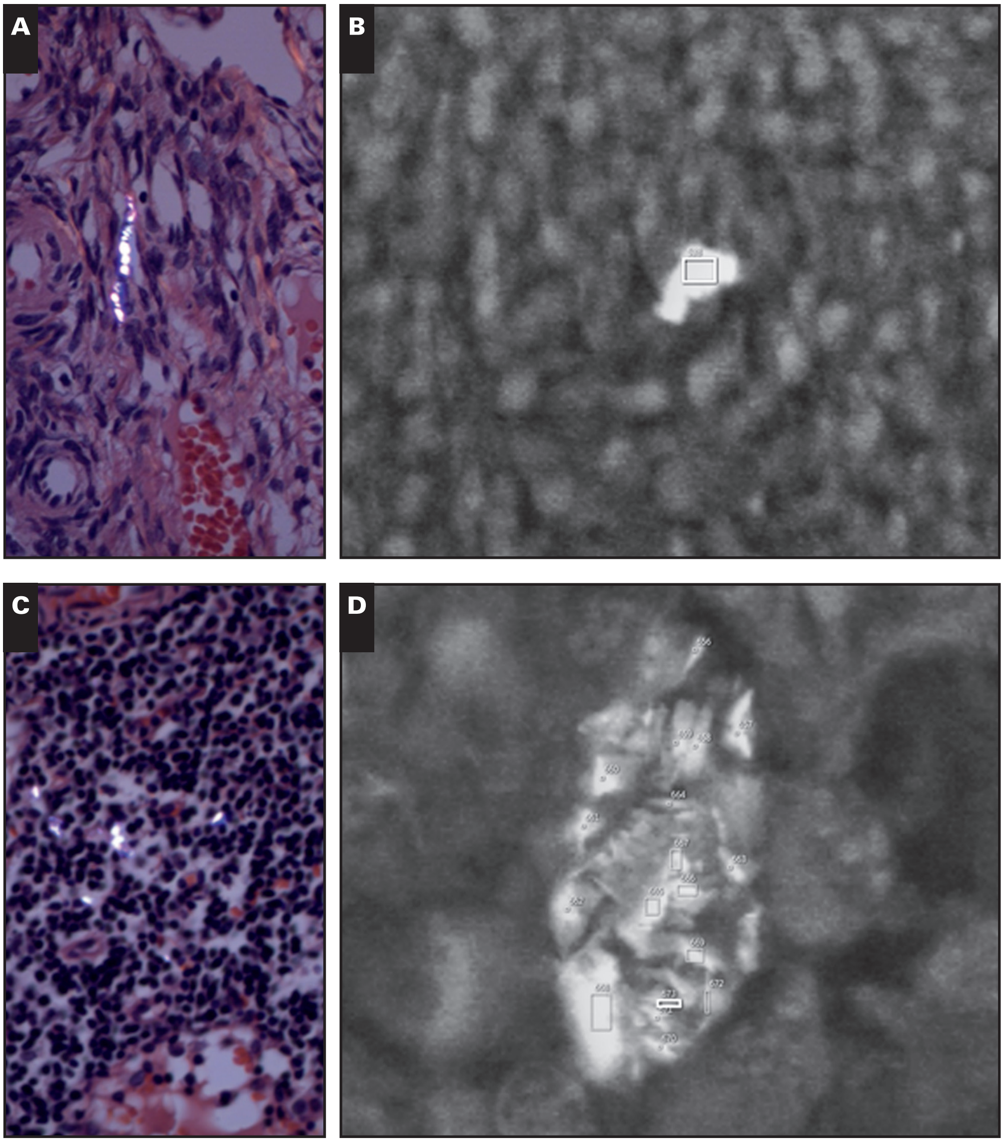

Image 7 Representative photomicrograph for patient 4. **A**, Tissue of the right ovary with a string-like arrangement of multiple birefringent particles (1-5 μm in greatest dimension) within ovarian stromal tissue (H&E, ×400). **B**, Scanning electron microscopy (SEM) showing an irregularly shaped particle, which in backscatter mode is about 6 μm in diameter. Energy-dispersive X-ray analysis of this particle showed the typical spectrum of talc (Image 1) (×1,000). **C**, Right pelvic lymph node tissue with approximately eight birefringent particles (each ~2 μm in greatest dimension or less) visible in the same plane of focus with the cells of the lymph node. Many of these particles are clearly within macrophage cells in the lymph node. **D**, SEM of the same right pelvic lymph node tissue (but a different section) showing numerous backscattered electron-positive particles within the cytoplasm of a macrophage, similar to the light microscopic morphology in **C**. These particles had the characteristic spectrum of talc (Image 1) (×1,000).

as readily as macrophages that have ingested granite or control macrophages, which may be why we also observe talc particles in tissue outside of phagocytic cells. This apparent initial avidity of macrophages for talc is consistent with the morphologic findings in our case series and may help explain the inflammatory potential of talc. Full reviews of macrophage biology and inflammatory responses are available in the literature, including the phenomena of reactive oxygen species generation and opsonization.[28-31] Talc may remain long after the initial inflammatory response has run its course, as evidenced by studies showing that talc has a slow dissolution rate in tissue.[24] In addition to the macrophage activity described earlier, mixed inflammatory infiltrates were sometimes seen in our talc-containing cases, for example, in the fallopian tube in patient 5 (Image 6) and the ovary in patient

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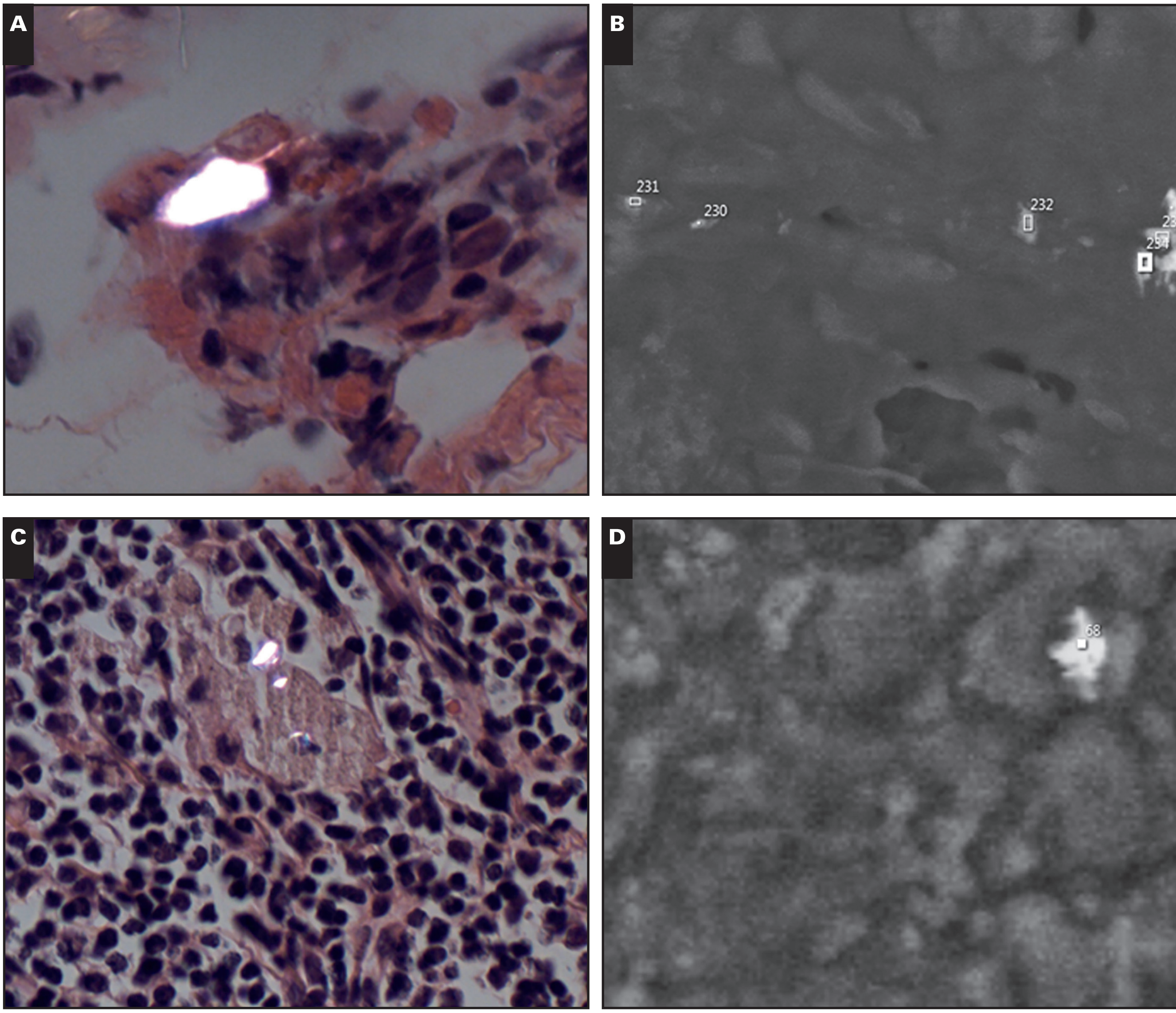

Image 8 Representative photomicrograph for patient 5. **A**, Birefringent particle approximately 7 µm in diameter, found in the soft tissues around the fallopian tube, and associated with chronic inflammation (H&E, ×400). **B**, Scanning electron microscopy (SEM) showing numerous backscattered electron-positive particles within the soft tissues around the same fallopian tube tissue as in **A** but a different tissue section (×500). **C**, Two birefringent particles within a left pelvic lymph node (H&E, ×400). **D**, SEM of the same pelvic lymph node tissue as in **C**, showing an irregularly shaped, backscattered electron-positive particle less than 5 µm in diameter, which showed the characteristic spectrum of talc.

2 (Image 3). The understanding of talc's ability to induce inflammation is well established.[32]

Through the migration of particles to lymph nodes as well as to other pelvic sites, the morphologic findings in our study indicate the likely importance of lymphatic pathways in the migration of talc. Talc may access lymphatics directly in the perineum (its typical initial exposure location) or at any point in its ascent through the genitourinary tract toward the fallopian tubes and ovaries. Among other possible mechanisms, this might occur through erosions in the superficial epithelial surface, thereby exposing the lymphatic channels directly underneath. Once talc particles reach ovaries and/or pelvic region lymph nodes, they have access to a further network of lymphatics present in those locations, thus yielding further migration potential. One example would be talc migration to para-aortic nodes, which we have seen in one patient (not included as part of this series but included in a separate report[19]) and conceptually mirrors the clinical finding that ovarian serous carcinoma tends to

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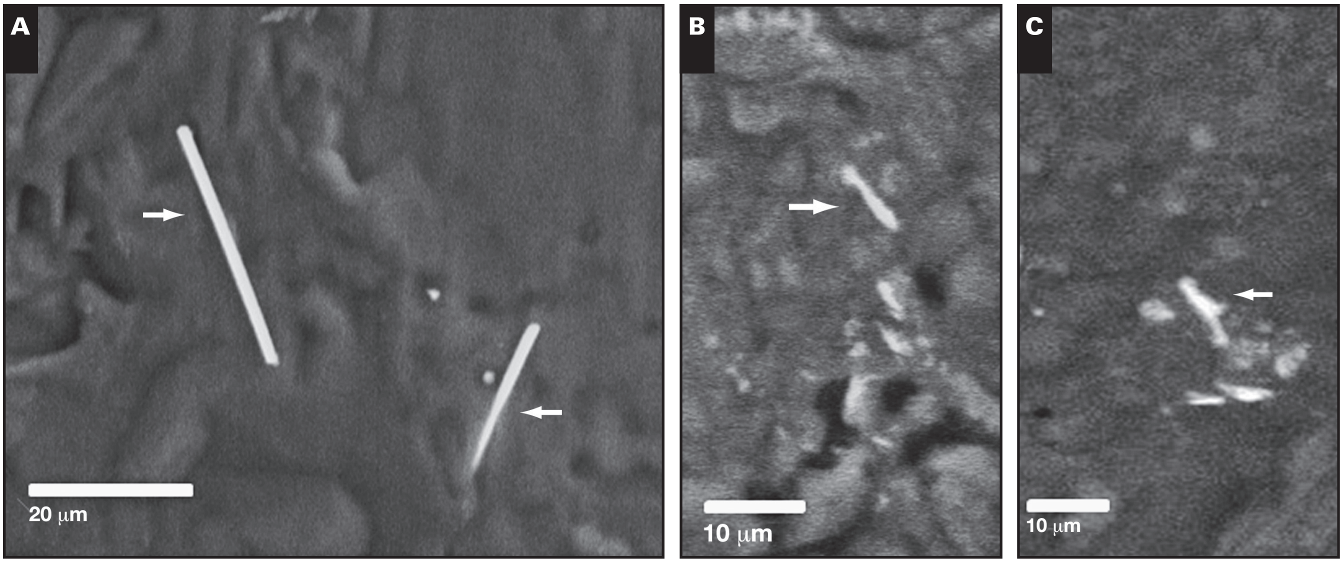

Image 9 Representative fibers and fiber-like particles (≥5:1 aspect ratio) found in our patient analysis (all photos are scanning electron microscopy with backscattered electron imaging, ×500). **A**, Long aspect ratio fibers (arrows) with a chemical signature of calcium (Ca), magnesium (Mg), aluminum (Al), iron, and silicon (Si) found in patient case 2 (right ovary). Fiber at right is seen to be extending into tissue where it disappears from view; thus, its aspect ratio may be higher than what is visible. For the total of four long aspect ratio fibers (10:1 or greater) that we found in our study, based on atomic weight percent calculations, average Mg/Si was 0.241 and Ca/Si was 1.03, where the respective ratios expected for tremolite are 0.542 and 0.357. Average Al/Si for the four fibers was 0.327, whereas no Al is expected for tremolite. **B**, Talc fiber-like particle (arrow) with an approximately 6:1 aspect ratio from case 2 (uterus). **C**, Talc fiber-like particle (arrow) from case 3 (uterus), with an approximately 6:1 aspect ratio. Particles in **B** and **C** had Mg-Si spectra with atomic weight percent ratios within 5% of the theoretical value of 0.649 for talc, similar to Image 1. In **C**, the other nearby backscattered electron-positive particles were also talc but did not meet the 5:1 aspect ratio threshold for a fibrous morphology.

metastasize early to para-aortic nodes preferentially over other node groups.[33] Theoretically, talc could even reach distant extrapelvic sites through further lymphatic spread and induce inflammatory reactions there, but in the women with ovarian malignancy who we have evaluated, this type of study opportunity has not arisen, simply because these extrapelvic tissues do not become available for examination as part of TAH/BSO surgery.

Besides the finding (with obvious implications) of exogenous material in lymph nodes, in our set of cases, evidence of lymphatic migration was seen in the distribution of birefringent material around small blood vessels in the uterine serosa (cases 2 and 3) and soft tissues at the periphery of the fallopian tube (cases 2 and 6). These areas are rich in lymphatics, and the clustering of exogenous material there is strongly suggestive of migration via such a route. Lymphatic vessels are highly distensible and compliant and have an elaborate pumping mechanism consisting of contractile lymphatic muscle cells and one-way valves that facilitate the transport of material (whether endogenous or exogenous) consistently via an anterograde flow route.[34] Initial lymphatics present in peripheral locations, such as those likely to be encountered in talc exposure, are typically tens of microns in diameter,[34] well within the range of the 1- to 10-μm size typically seen for talc particles in exposure settings and also consistent with the size of pathogens, malignant cells, and other materials the lymph system typically collects and transports.

Sentinel lymph node studies, although derived from oncology, offer insight into the migration potential of talc from the perineum or lower genital tract and help explain the peculiar idiosyncratic specificity of talc migration sites that is often seen in patient cases. The general principle from sentinel lymph node studies is that usually there is one node or at most a small group of nodes that represent the initial site of dissemination or metastasis in a given patient, among many nodes that in theory are part of the drainage basin,[35] and so if that sentinel node is free of metastases, then remaining nodes in the same nodal basin should be free also.[36] The lymphatic network of the ovary is known to be both rich and complex and subject to frequent remodeling based on the menstrual/hormonal cycle.[37] Based on studies of ovarian

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malignancy, the most common drainage sites from the ovary are the pelvic, paraaortic, and iliac lymph nodes,[38] so talc that migrates to the ovary would then be expected to have access to these lymph node groups through a similar mechanism. A further study by Vanneuville et al[39] using lymphoscintigraphy on 14 patients showed that ovarian lymphatic drainage may be age dependent, with premenopausal drainage likely to be both pelvic and para-aortic but postmenopausal drainage likely to be predominantly para-aortic. As for the lymphatic drainage basin for the uterus, pelvic and para-aortic nodes may become involved simultaneously, in contrast to those in the cervix, where pelvic nodes become involved first and then result in spread to para-aortic nodes.[40] Further sentinel lymph node studies have showed that within the uterus, upper and lower drainage pathways exist, with the former draining to external iliac and obturator lymph nodes and the latter draining to the internal iliac and presacral lymph nodes.[41] Furthermore, pelvic lymphatic pathways are frequently anastomosing, idiosyncratic, and subject to modification.[37,39,40]

Taking all of this together, it seems prudent to conclude that once talc gains access to the lymphatic system anywhere in the female genital tract, it could potentially be detected in any of the lymph node groups previously described for which metastases and sentinel lymph node tracers have been localized in the past, as well as in solid organ sites (ovary, fallopian tube, uterus), which contain efferent from those same lymphatic networks. This is entirely consistent with the spectrum of histologic findings that we report in this case series. It is also likely that patterns of talc dissemination, like patterns of lymphatic drainage and metastasis in other settings, are likely to be idiosyncratic and patient dependent, without clear explanations in most cases as to why a given patient localized foreign material in a particular node or site and not other sites, other than a given particular drainage pathway simply being what that patient's anatomy prefers. Other factors, such as the overall burden of exposure, the exact sites of exposure and the nature of the physical application, and the size distribution of the talc particle exposure, all also likely play roles in whether and where pelvic migration pathways develop.

Among the five patients in the main study, two had a history of tubal ligation (cases 2 and 3, Table 1). In theory, this should reduce the risk of ovarian carcinoma from talc exposure by blocking the latter's ascension to the ovaries through the reproductive tract, thus mitigating inflammatory effects. In fact, some but not all studies have shown an increased risk in malignancy from talc use in women who have not undergone tubal ligation.[5,42,43] What is interesting is that the two patients in our study with tubal ligations had numerous talc particles in a strongly lymphovascular distribution in their uterine serosal areas (Tables 2 and 3 and Image 3), with patient 2 also showing abundant talc in pelvic region lymph nodes (nodes were not sampled in patient 3). Although the numbers here are too small to draw definitive conclusions, an interesting possibility is that blockage of the reproductive tract passage may lead to countervailing greater access of talc to the lymphatic system, especially if exposure levels are high.

Talc found in our study was usually polygonal and nonfibrous; nevertheless, 18 fiber-like talc particles were found across the main part of the study, with an aspect ratio of 5:1 or more. These were typically found in areas with large collections of talc particles overall (eg, macrophages, lymph nodes) and so most likely simply represent one end of the size distribution of naturally heterogeneous particles in size and shape. Only four long aspect fibers (≥10:1) were found, and these were nonasbestos.

The expanded understanding of talc's biologic potential, as evident in this set of cases, has implications for surgical pathologists who review TAH/BSO specimens from patients with ovarian carcinoma. If a history of talc use is known or suspected, it may be prudent to examine with polarizing light microscopy the range of tissue types studied in this case and not simply the ovaries (although the latter is indeed a prudent place to start, especially if benign residual stroma can be found). If birefringent particles are identified in the tissue, the corresponding slides and blocks can then be referred for SEM/EDX analysis for confirmation. Based on our surgical pathology experience, the macrophage, giant cell, and chronic inflammatory infiltrates seen in some of our talc-containing cases by light microscopy are unlikely to be pathognomonic alone. Thus, while their presence is of interest, especially in the right clinical setting (ie, history of talc use), the auxiliary studies described here would be needed. Our concomitant study of six patient controls supports the contention that talc is rarely found in the pelvic tissues of nonexposed patients. The findings in the main study, especially balanced against the control tissue findings, further support the contention that unexpected or unexplained inflammatory infiltrates (especially chronic or macrophage-rich), combined with birefringent material on polarized microscopy, should prompt SEM/EDX for confirmation of talc (or, if not talc, whatever the exogenous substance may be).

Conclusion

The existence of morphologically demonstrated talc in multiple pelvic organ sites, including pelvic tissues and lymph nodes simultaneously, which is reported here in

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multiple patients, has not been reported to our knowledge. Given the ongoing concerns regarding talc, particularly with regard to its epidemiologic association with ovarian cancer, these findings are important and offer new insight into the biologic potential of talc, its inflammatory potential, and its migration via pelvic lymphatics from the perineum. Along with the available epidemiologic studies and the few previous morphology-based reports, the findings suggest that clinicians may want to closely examine pelvic organs and lymph nodes (when made available through surgical resection) for talc in patients with ovarian carcinoma and a history of perineal talc use. The index of suspicion is especially high in cases with birefringent material (by polarizing light microscopy) and unexplained chronic or macrophage-rich inflammatory infiltrates in pelvic tissues.

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